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The complex nature of the genetic events that trigger the development of breast cancer remain to be indentified. Our laboratory has developed and refined a retrovirus-based gene transfer assay to identify activated oncogenes in breast cancer. Progress during the past year has involved: (1) development of better functional screens for activated oncogenes, (2) evaluation of protocols to improve the rescue of cDNAs from the transformed cell populations, and (3) preliminary analyses of isolated transforming genes. We have generated several retrovirus-based cDNA expression libraries that represent genes expressed in noninvasive (T47D) or invasive (MDA-MB4682, BT549 and Hs578T) human breast cancer cell lines. The T47D library has been introduced into Rat-1 rodent fibroblasts and RIE-1 rat intestinal epithelial cells and transforming activity was then assayed for. Whereas empty retrovirus vector-infected cells did not show any transforming activity, cultures infected with breast tumor cDNAs showed the appearance of over 100 colonies of transformed cells. Two isolates from our analyses encode proteins with known growth-promoting functions: the Raf-1 serine/threonine kinase and the fibroblast growth factor receptor 2. A third, syntaxin 6, a protein involved in intracellular vesicular transport has not bee linked previously to oncogenesis.

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## Introduction

Breast cancers arise because of mutations in "good genes gone bad". The complex nature of the genetic events that trigger the development of breast cancer remain to be defined. While some of these genes have been identified, many remain to be discovered. Until the genes involved in breast cancer are identified and their mode of action understood, our progress in the development of diagnostic markers and anti-neoplastic drugs for breast cancer treatment will be hindered. Thus, there is a clear and urgent need to identify genes whose aberrant functions contribute to breast cancer development. Only when a detailed understanding of the genes that go awry in the cancer cell is achieved, can we make significant progress in the diagnosis and treatment of breast cancer. Our ability to dissect the genetic basis of breast cancer is limited by the complex nature of the disease and by the tools and scientific technology available to researchers.

Recently developed microarray analyses have provided powerful approaches to identify genes whose expression are deregulated in human breast cancers (1). However, the functional relevance of the identified genes remains to be determined. This can be a daunting task, since hundreds of genes are identify by these approaches. Gene transfer discovery studies have been a powerful approach for identifying some of the key genes important in the development of some cancers (2,3). A critical advantage of these types of approaches is that function is used to identify the genes involved. However, these approaches have met with limited success in the study of breast cancer. Our laboratory and others have developed and refined these gene transfer techniques to a new level (3) where we feel that they are now ready to be applied to the study of breast cancer. Based on our application of these techniques to other cancers, we are confident that we will identify novel genes important for breast cancer development. The identification of such genes will aid in the development of new diagnostic markers for the early detection of breast cancer. They may also establish new targets for the development of novel anti-cancer drugs for breast cancer treatment.

## Body

**A. Generation and screening of retrovirus cDNA expression libraries** - A serious potential limitation of previous screening efforts for novel oncogenes has been the use of the NIH 3T3 mouse fibroblast cell line. First, these cells are prone to a high frequency of spontaneous transformation. In our initial screens using a pCTV3 retrovirus vector-based cDNA expression library (4) made from an invasive breast carcinoma cell line, we did identify over 100 foci of transformed cells using the NIH 3T3 focus formation assay. Over 50 of these foci were isolated using cloning cylinders, expanded, and frozen down. However, the parallel control experiment using the empty pCTV3 retrovirus also resulted in an unexpectedly high frequency of spontaneous foci of transformed cells. While the number found on the control dishes was clearly several fold lower than that seen on the cDNA expression library dishes, this indicated that a good percentage of these foci probably represented false positive clones. Nevertheless, we did pursue the analyses of some of these isolates to identify the cDNA sequences involved. This work unfortunately did not lead to any identified transforming sequences.

To overcome this limitation of NIH 3T3, we have extended our analyses to use Rat-1 fibroblasts. In contrast to NIH 3T3 cells, Rat-1 cells have a very low rate of spontaneous transformation. However, relative to NIH 3T3 cells, Rat-1 cells are poor recipients for transfected DNA. The use of retrovirus infection as a means to introduce DNA should overcome this limitation. We performed trial experiments with the empty pCTV3 vector, as well as one that encodes the green fluorescent protein (GFP). We found that the rate of spontaneous foci of transformed cells is essentially nonexistent with the empty vector. We also found a high rate of infection of Rat-1 cultures, as indicated by >80-90% of the cells expressing GFP. These trial analyses prompted our use of Rat-1 cells for our screens. We have screened one breast library using Rat-1 cells and have isolated over 50 transformed foci of cells. An example of the type of foci seen in Rat-1 cells is shown in Fig. 1 (P2Y2R is a G protein-coupled receptor our lab discovered in a screen of patient-derived acute leukemia cells).

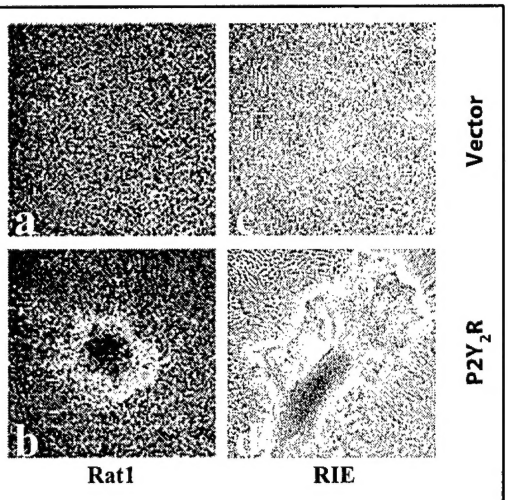



**Figure 1. $P2Y_2R$ transforms Rat1-Fibroblasts and RIE cells.** Expression of $P2Y_2R$-pBABE causes the formation of foci in both Rat1-Fibroblasts (b) and RIE cells (d). The vectors do not show such transformation (a) & (c).

A second limitation of using NIH 3T3 cells, is the epithelial cell origin of breast cancers. Therefore NIH 3T3, as well as Rat-1, fibroblast cells may not be the most appropriate host for the detection of epithelial derived oncogenes. Indeed, we have recently performed experiments on several epithelial cell lines (IEC-6, RIE-1, C127, MCF-10A, etc.) that provide compelling evidence that fibroblast and epithelial cells are sensitive to distinct oncogenes. Therefore, in addition to the Rat-1 cells, we have evaluated RIE-1 cells as a recipient for screening. These cells are sensitive to one-hit transformation by activated Ras and a variety of oncogenes (Fig. 2). We also found that they are not susceptible to spontaneous transformation when we infected them with the empty pCTV3 retrovirus vector and they were infected at a high frequency as measured by a high efficiency of GFP expression. Hence, we have used both Rat-1 and RIE-1 for our transformation assays concurrently in screening the breast cancer cell expression libraries.

**B. Isolation of transforming sequences** – We have found that the generation of the cDNA expression libraries, when coupled with the very clean transformation assays using Rat-1 or RIE-1 cells, has made it very efficient in detecting transforming activity. Therefore, the isolation of transformed cell populations from our screens has been quite efficient and we have isolated over 50 foci of transformed cells, and we have expanded these populations and have stored each in liquid nitrogen.

The next step, the isolation of the retrovirus-associated transforming gene, has proved to be the rate-limiting step in the entire process. We have struggled with this for many months. The basic aspect of this step is to use oligonucleotide primers that correspond to retrovirus sequences that flank the inserted cDNA sequences in PCR-

mediated DNA amplification using total genomic DNA isolated from each transformed cell population. As shown in Fig. 2, our initial attempts met with difficulty in getting clear amplication of sequences, as indicated by a clear fragment when the PCR products were analyzed in agarose gels. This has made it difficult to decide which isolates to pursue for subcloning and further analyses.

We tried initially to improve the quality of the isolated genomic DNA, assuming that this may have been a main limitation of the quality of the PCR-amplified sequences. We tried a variety methods; however, none provides use with a significant improvement in our ability to get clean DNA fragments from our PCR amplifications.

Recently, we have tried different enzymes and conditions for the PCR amplification. The data shown in Fig. 2 compares the PCR products using two different approaches and shows improvements in rescuing the cDNA clone back out from genomic DNA of the 'hits'. The original PCR reaction (for rescuing cDNA) was amplified with Pfu Turbo DNA Polymerase (from Stratagene) using 50 ng of genomic DNA. On the average, our success with PCR-mediated amplification from genomic DNA (only with regards to genomic DNA obtained from the library screens) has been about 1 out of 10 clones. So we've actually had more hits that remain to be identified because we can't always rescue the clones. Recently, we switched to Tth DNA Polymerase (from Clonetech). This enzyme works under reaction conditions that are similar to that for Pfu except with the addition of GC-Melt (a solvent that is used to melt and keep denatured genomic DNA, especially the GC rich regions, remain single strandedlonger to give primers a chance to anneal to the template). Use of this solvent produced single specific amplified products as opposed to multiple bands or sometimes smears. With Pfu, it was unclear what fragment was best to isolate for subcloning and also whether the same sized DNA insert was present in the different isolates. With Tth, it was much more obvious what the insert fragment entailed. After sequence analyses of several isolated sequences, we found that all encode for the fibroblast growth factor receptor 2 (FGFR2).

We subcloned the cDNA sequence for FGFR2 into the pBabe-puro retrovirus vector and reintroduced the gene back into Rat-1 cells to verify the transforming potential of this sequence (Fig. 3). The induction of foci of transformed cells verified that FGFR2 was likely the transforming activity detected in the screen using T47D-derived cDNA sequences. FGFR2 has been linked to breast cancer development (6-8). A comparison of the sequence of the isolated FGFR2 gene with that of the wild type sequence indicated no mutations. Thus, FGFR2 mediated transformation by overexpression. Therefore, future studies will determine if FGFR2 protein and signaling is upregulated in T47D cells and whether inhibition of FGFR2 function will impair the transformed and tumorigenic growth of T47D cells.

**Key Research Accomplishments**

- Generation of retrovirus cDNA expression libraries that represent the entire complexity of genes expressed in various human breast cancer cell lines
- Establishment of Rat-1 and RIE-1 as functional screens for oncogenes – the lack of background spontaneous foci in these screens has made a great impact on our ability

to isolate transforming sequences – we now have more isolates than we can effectively process

- Refinement of the PCR-based approaches to isolate and subclone the transforming sequences – this has greatly improve the rate-limiting step in our analyses
- Isolation and analyses of Raf-1 and FGFR2 as transforming sequences expressed in T47D cells

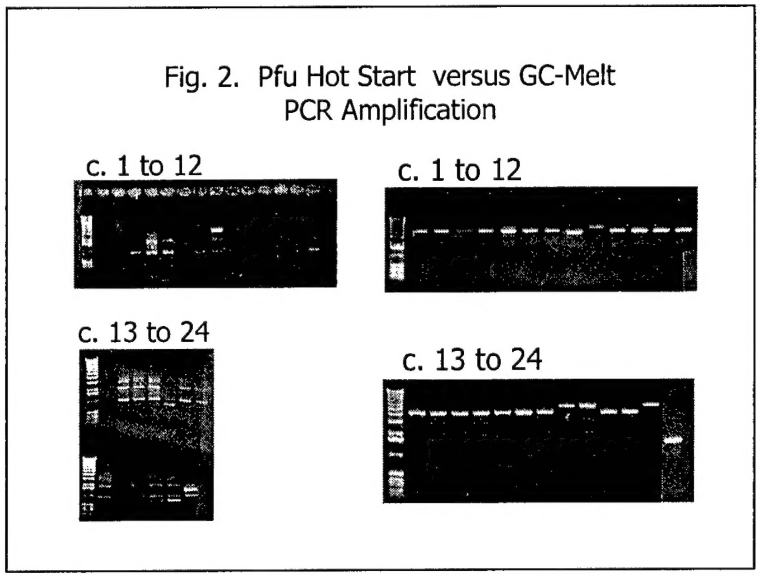



Fig. 2. Pfu Hot Start versus GC-Melt PCR Amplification

## Reportable Outcomes

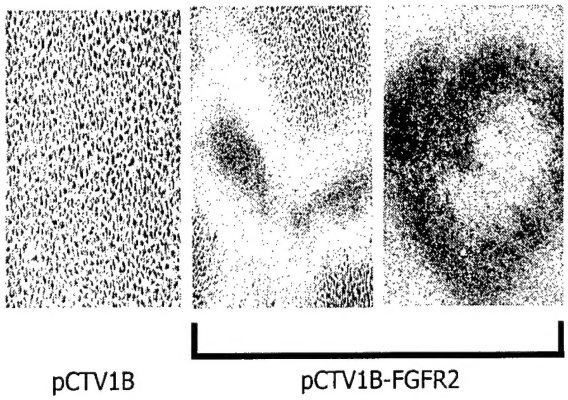
* Abstract and presentation for upcoming Era of Hope Meeting, Orlando, FL 2002

## Conclusions

Overall, this project is extremely labor-intensive. Hence, a large component of our studies in these library screens has been technology development. We felt that an emphasis on improving the screen, both in accuracy and in efficiency and a reduction in labor, would pay off in the long run. The development of Rat-1 and RIE-1 cells as screens has been a significant step on our studies, since it now gives us greater confidence that when we isolate a transformed foci, the introduced retrovirus-associated sequence is most likely responsible for the transforming activity. When we started with the NIH 3T3 screening, and identified many transformed foci, the analyses of these foci were difficult because we were not confident that the isolated gene was actually a transforming gene. We now have over 50 foci of transformed Rat-1 or RIE-1 cells that have been isolated and are now ready for sequence isolation and analyses. The next key development involved finally identifying a PCR-based approach that yielded clean and efficient amplification of the retrovirus-associated sequences. This has been the most frustrating aspect of these studies, has delayed our progress for many months, but we now feel that we have made a major improvement in this step. We are only now taking advantage of this important breakthrough for our studies to

isolate and identify transforming genes. Our first two candidates encode known signaling proteins, the Raf-1 serine/threonine kinase, and the FGFR2 receptor tyrosine kinase. The first is surprising, because to date, Raf-1 can be activated only by structural mutations (5). The Raf-1 we isolated is wild type in sequence. The second is intriguing in light of many observations that FGFRs are amplified and involved in breast cancer development (6-8). So, in summary, we hope that these studies will now move from technology development to science and gene discovery and analyses in the following year. One future direction will be to see if Raf-1 and FGF2 are commonly overexpressed in breast cancers and whether this overexpression is functionally important. The second will be the isolation of more transforming sequences and the emphasis on novel gene targets. We have also isolated syntaxin-6 as a transforming gene in our screen. To date, there is no evidence for syntaxin-6 in cancer. Therefore, we will first verify that syntaxin-6 exhibits transforming activity in transformation assays using rodent or human mammary epithelial cells. We will also evaluate the level of mRNA and protein expression in T47D cells. Finally, we will determine if dominant negative mutants of syntaxin will impair the growth of T47D cells in vitro (soft agar growth) or in vivo (tumor formation).

Fig. 3. FGFR2 Focus Formation in Rat-1 Cells



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**Appendices** - None